APPLICATION FOR PATENT

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Title:

ENHANCING INORGANIC CARBON FIXATION BY PHOTOSYNTHETIC ORGANISMS

This is a continuation of U.S. Patent Application No. 09/332,041, filed June 14, 1999.

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to a method of enhancing inorganic carbon fixation by photosynthetic organisms, nucleic acid molecules for effecting the method, and transformed plants characterized by enhanced inorganic carbon fixation.

Photosynthesis is a process executed by photosynthetic organisms by which, inorganic carbon (Ci), such as CO_2 and HCO_3^-, is incorporated into organic compounds using the energy of photon radiation. Photosynthetic organisms, such as, soil grown and aquatic plants and

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The present invention relates to a method of enhancing inorganic carbon fixation by photosynthetic organisms, nucleic acid molecules for effecting the method, and transformed plants characterized by enhanced inorganic carbon fixation.

Photosynthesis is a process executed by photosynthetic organisms by which, inorganic carbon (Ci), such as CO_2 and HCO_3^-, is incorporated into organic compounds using the energy of photon radiation. Photosynthetic organisms, such as, soil grown and aquatic plants and

cyanobacteria (blue-green algae), depend on the organic compounds produced via photosynthesis for sustenance and growth.

The rate of photosynthesis is determined by several parameters which include but are not limited to, CO_2 concentration, O_2 concentration, temperature, light intensity, and the water balance in the case of soil grown plants.

Of the above mentioned parameters photosynthesis is largely influenced by the rate with which the carboxylating enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase, (rubisco) can fix available CO_2. The rate of CO_2 fixation depends on the concentration of CO_2 and to a lesser extent on the concentration of O_2 which are available to rubisco, since CO_2 fixation competes with photorespiration, which is the process of O_2 fixation by rubisco. Furthermore, when photosynthesis is rate-limited by the supply of CO_2, the imbalance between the light energy input and its dissipation via CO_2 fixation, leads to photodynamic damage to the photosynthetic reaction centers (photoinhibition).

Since rubisco posseses a very slow turnover rate, in order to meet the energy requirements of photosynthetic organisms it needs to be present in abundance within the photosynthetic cells thereof

(approximately 50 % of the soluble protein). The abundance of rubisco leads to deleterious effects to the energetic balance of photosynthetic cells since most available resources of these cells must be allocated to the production of rubisco.

To overcome the problems associated with inefficient CO_2 fixation at low CO_2 concentrations, many photosynthetic organisms have evolved various mechanisms for concentrating the inorganic carbon exposed to rubisco. Such mechanisms are found in certain higher plants (belonging to the C4 group, including maize and sorghum) and in aquatic photosynthetic organisms. In C4 plants, differential expression and tight regulation of several genes enables cooperation between the mesophyll and bundle sheath cells leading to elevated CO_2 concentration in the latter. An initial carboxylation reaction, where HCO_3^- serves as the substrate, occurs in the mesophyll cells. The product is then transferred to the bundle sheath cells where it is decarboxylated (releasing the CO_2 fixed in the mesophyll cells) in close proximity to rubisco (confined to these cells). C3 plants, to which most of the crop plants belong, are unable to concentrate CO_2 at the site of rubisco and therefore grow poorly (compared to C4 plants) under water-limiting conditions. Due to

the large number of genes involved, and to the complexity of their tight, spatial regulation, in the operation of the C4 mechanism, the introduction of the whole C4 CO_2 concentrating mechanism to C3 plants is presently impossible.

Many aquatic photosynthetic microorganisms possess inducible mechanisms that concentrate CO_2 at the carboxylation site, compensating for the relatively low affinity of rubisco for its substrate, thus allowing acclimation to a wide range of CO_2 concentrations [25]. The presence of membrane-located mechanisms for inorganic carbon (Ci) transport are central to these concentrating mechanisms.

Photosynthetic microorganisms including cyanobacteria are also capable of acclimating to a wide range of CO_2 concentrations. The process of acclimation is mediated via changes, effected at various cellular levels, which include modulation of gene expression involved in the operation of the CO_2 concentrating mechanism (CCM) [1-5]. This mechanism enables these photosynthetic microorganisms to raise the CO_2 level at the carboxylation site thus overcoming the large (5 to 20-fold) difference between the $K_m(CO_2)$ of rubisco and the concentration of dissolved CO_2 when at equilibrium with the surrounding atmosphere. In

cyanobacteria, the components of the CCM include energy-dependent HCO_3^- transport, CO_2 conversion to HCO_3^- [3] and highly organized structures, termed carboxysomes, where carbonic anhydrase catalyzes the formation of CO_2 from HCO_3^- in close proximity to rubisco [1-3]. The activity of the CCM increases dramatically following transfer from high to low CO_2 concentrations mainly due to changes in the Ci transport capabilities and an increase in the number of carboxysomes [3, 6, 7]. Some of the genes involved in the operation of the CCM were identified with the aid of high-CO_2-requiring mutants but there is little information on those directly involved in HCO_3^- uptake [3, 4, 8]

The ability to concentrate CO_2 provides distinct advantages to photosynthetic organisms. The photosynthetic rate of such CO_2 concentrating organisms is not severely affected by lower CO_2 concentrations and as a result, the growth and productivity of such organisms are not severely limited by the environmental concentration of CO_2, and by water, in the case of soil grown plants.

Therefore, it is highly desirable to enhance CO_2 fixation in non CO_2 concentrating photosynthetic organisms, such as C3 plants, since in

all probability, such an enhancement would directly result in improved growth and productivity.

There are two possible biotechnological approaches with which an enhanced rate of CO_2 fixation can be achieved.

One such approach involves the manipulation of rubisco by site directed mutagenesis in order to raise both its affinity and specificity to CO_2 (compared with O_2) and to further enhance its turnover rate. Although numerous studies were conducted in order to isolate a rubisco mutant which posseses the above mentioned improvements at present no such rubisco mutants have been isolated.

Another approach for enhancing CO_2 fixation involves raising the concentration of CO_2 outside the cells of higher plants. In higher plants the concentration of CO_2 in the air spaces within the leaves is determined by the diffusional flux of CO_2 through the stomata from the surrounding air via the unstirred layer around the leaves. The stomatal conductance for CO_2 is largely determined by the water balance of the plants. Modulation of stomatal opening by water availability is exercised by plants in order to combat water stress. Stomatal closure, in order to minimize water loss under conditions of water stress, generates a

significant resistance to CO_2 diffusion leading to a sharp decline in CO_2 fixation. Although raising the concentration of CO_2 in a closed environment, such as a greenhouse, is commonly practiced in order to raise the diffusional flux of CO_2 and as such, raise plant productivity, such a practice however, is not applicable to outdoor grown plants.

Increasing stomatal conductance can theoretically serve to raise plant productivity, but at present, viable mechanisms for enhancing the stomatal CO_2 conductivity have not been proposed.

Thus, at present, both of the above mentioned approaches are theoretical and as such cannot be applied to render photosynthetic organisms, such as C3 plants, more efficient at fixing CO_2.

There is thus a widely recognized need for, and it would be highly advantageous to have, a method of enhancing the CO_2 fixation in photosynthetic organisms, such as higher plants, especially C3 plants.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of enhancing inorganic carbon fixation by a photosynthetic

organism, the method comprising the step of transforming cells of the photosynthetic organism with an expressible polynucleotide encoding a polypeptide having a bicarbonate transporter activity. Preferably, the polynucleotide further includes a plant promoter.

According to another aspect of the present invention there is provided a nucleic acid molecule for enhancing inorganic carbon fixation by a photosynthetic organism, the nucleic acid molecule comprising a polynucleotide encoding a polypeptide having a bicarbonate transporter activity. Preferably, the nucleic acid molecule further comprising a plant promoter being upstream to the polynucleotide effective in expressing the polypeptide.

According to yet another aspect of the present invention there is provided a transformed photosynthetic organism comprising the nucleic acid molecule described herein.

According to further features in preferred embodiments of the invention described below, the step of transforming the cells of the photosynthetic organism with the expressible polynucleotide encoding the bicarbonate transporter is effected by a method selected from the group consisting of genetic transformation and transient transformation.

According to still further features in the described preferred embodiments the genetic transformation is effected by a method selected from the group consisting of Agrobaterium mediated transformation, electroporation and particle bombardment.

According to still further features in the described preferred embodiments the transient transformation is effected by a method selected from the group consisting of viral transformation, electroporation and particle bombardment.

According to still further features in the described preferred embodiments the polynucleotide includes (i) a nucleic acid sequence corresponding to at least a portion derived from SEQ ID NO:2, the portion encodes the protein having the bicarbonate transporter activity; (ii) a nucleic acid sequence at least 60 % identical to the portion, as determined using the Blast software where gap penalty equals 10 for existence and 10 for extension, average match equals 10 and average mismatch equals -5; (iii) a nucleic acid segment hybridizable with the portion under hybridization conditions of hybridization solution containing 10 % dextrane sulfate, 1 M NaCl, 1 % SDS and 5 x 10^6 cpm ^{32}p labeled probe, at 65 °C, with a final wash solution of 0.2 x SSC and

0.1 % SDS and final wash at 65 °C; (iv) a man induced variation of the portion; or (v) a naturally occurring variation of the portion.

According to still further features in the described preferred embodiments the polypeptide is at least 70 % homologous to SEQ ID NO:3 or a portion thereof having the bicarbonate transporter activity as determined using the Blast software where gap open penalty equals 11, gap extension penalty equals 1 and matrix is blosum62.

According to still further features in the described preferred embodiments the photosynthetic organism is a plant.

According to still further features in the described preferred embodiments the plant is a C3 plant.

According to still further features in the described preferred embodiments the C3 plant is selected from the group consisting of tobacco, tomato, soybeans, potato, cucumber, cotton, wheat, rice and barley.

According to still further features in the described preferred embodiments the plant is a C4 plant.

According to still further features in the described preferred embodiments the C4 plant is selected from the group consisting of corn, sugar cane and sorghum.

According to still further features in the described preferred embodiments the organism is characterized by a photosynthetic rate at least 10 % higher as compared to a control non-transformed organism under otherwise identical conditions.

According to still further features in the described preferred embodiments the plant promoter is selected from the group consisting of a constitutive plant promoter, a tissue specific plant promoter and an inducible plant promoter.

According to still further features in the described preferred embodiments (i) the constitutive plant promoter is independently selected from the group consisting of CaMV35S plant promoter, CaMV19S plant promoter, FMV34S plant promoter, sugarcane bacilliform badnavirus plant promoter, CsVMV plant promoter, *Arabidopsis* ACT2/ACT8 actin plant promoter, *Arabidopsis* ubiquitin UBQ1 plant promoter, barley leaf thionin BTH6 plant promoter, and rice actin plant promoter; (ii) the tissue specific plant promoter is independently selected from the group

consisting of bean phaseolin storage protein plant promoter, DLEC plant promoter, PHSβ plant promoter, zein storage protein plant promoter, conglutin gamma plant promoter from soybean, AT2S1 gene plant promoter, ACT11 actin plant promoter from *Arabidopsis*, napA plant promoter from *Brassica napus* and potato patatin gene plant promoter; and (iii) the inducible plant promoter is independently selected from the group consisting of a light-inducible plant promoter derived from the pea rbcS gene, a plant promoter from the alfalfa rbcS gene, DRE, MYC and MYB plant promoters which are active in drought; INT, INPS, prxEa, Ha hsp17.7G4 and RD21 plant promoters active in high salinity and osmotic stress, and hsr203J and str246C plant promoters active in pathogenic stress.

According to still further features in the described preferred embodiments the polynucleotide further includes a sequence element selected from the group consisting of a nucleic acid sequence encoding a transit peptide, an origin of replication for propagation in bacterial cells, at least one sequence element for integration into a plant's genome, a polyadenylation recognition sequence, a transcription termination signal, a sequence encoding a translation start site, a sequence encoding a

translation stop site, plant RNA virus derived sequences, plant DNA virus derived sequences, tumor inducing (Ti) plasmid derived sequences and a transposable element derived sequence.

The present invention provides new horizons with respect to crop yields, especially of C3 plants grown under low CO_2 and/or under limited water availability, because it accelerates the transport of bicarbonate into plant cells and eventually the chloroplasts to thereby improve the photosynthetic process and as a result carbon fixation.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 is a schematic representation of a genomic region in *Synechococcus* sp. PCC 7942 where an insertion (indicated by a star) of an inactivation library fragment led to the formation of mutant IL-2. DNA sequence is available in the GenBank, Accession number U62616. Restriction sites are marked as: A - *Apa*I, B - *Bam*HI, Ei - *Eco*RI, E -

*Eco*RV, H - *Hinc*II, Hi - *Hind*III, K - *Kpn*I, M - *Mfe*I, N - *Nhe*I, T - *Taq*I. Underlined letters represent the terminate position of the DNA fragments that were used as probes. Relevant fragments isolated from an EMBL3 library are marked E1, E2 and E3. P1 and P2 are fragments obtained by PCR. Triangles indicate sites where a cartridge encoding Kan^r was inserted. Open reading frames are marked by an arrow and their similarities to other proteins are noted. Sll and slr (followed by four digits) are the homologous genes in *Synechocystis* PCC 6803 [23]; YZ02-myctu, Accession No. Q10536; ICC, Accession No. P36650; Y128-SYNP6, Accession No. P05677; YGGH, Accession No. P44648; Ribosome binding factor A homologous to sll0754 and to P45141; O-acetylhomoserine sulfhydrylase homologous to sll0077 and NifS. ORF280 started upstream of the schematic representation presented herein.

FIG. 2 shows nucleic acid sequence alignment between ORF467 (ICTB, SEQ ID NO:2) and slr1515 (SLR, SEQ ID NO:4). Vertical lines indicate nucleotide identity. Gaps are indicated by hyphens. Alignment was performed using the Blast software where gap penalty equals 10 for

existence and 10 for extension, average match equals 10 and average mismatch equals -5. Identical nucleotides equals 56 %.

FIG. 3 shows amino acid sequence alignment between the IctB protein (ICTB, SEQ ID NO:3) and the protein encoded by slr1515 (SLR, SEQ ID NO:5). Identical amino acids are marked by their single letter code between the aligned sequences, similar amino acids are indicated by a plus sign. Alignment was performed using the Blast software where gap open penalty equals 11, gap extension penalty equals 1 and matrix is blosum62. Identical amino acids equals 47 %, similar amino acids equals 16 %, total homology equals 63 %.

FIGs. 4a-b are graphs showing the rates of CO_2 and of HCO_3^- uptake by *Synechococcus* PCC 7942 (2a) and mutant IL-2 (2b) as a function of external Ci concentration. The rates were assessed from measurements during steady state photosynthesis using a membrane inlet mass spectrometer (MIMS) [6, 7, 22].

FIG. 5 presents DNA sequence homology comparison of a region of *ictB* found in *Synechococcus* PCC 7942 and in mutant IL-2. This region was duplicated in the mutant due to a single cross-over event. Compared with the wild type, one additional nucleotide and a deletion of

six nucleotides were found in the *Bam*HI side, and 4 nucleotides were deleted in the *Apa*I side (see Figure 1). These changes resulted in stop codons in *IctB* after 168 or 80 amino acids in the *Bam*HI and *Apa*I sides, respectively. The sequence shown by this Figure starts from the 69th amino acid of *ictB*.

FIG. 6 is a photograph of a RNA gel blot showing expression of *ictB* RNA in transgenic Arabidopsis plants. RNA was isolated from wild-type (WT) and 3 independent transgenic plants and subjected to RNA gel blot analysis with a probe for *ictB*. WT plants were found not to contain transcript(s) that hybridized with the ictB probe. The transgenic lines shown in this Figure were used for the CO_2 exchange analysis presented in Table 2.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a method which can be used to enhance inorganic carbon fixation by photosynthetic organisms. The present invention is further of nucleic acid molecules which can be used

to enhance inorganic carbon fixation by photosynthetic organisms. The present invention is further of transformed plants characterized by enhanced inorganic carbon fixation. More specifically, the present invention employs an overexpressed bicarbonate transporter for enhancing carbon fixation in particular C3 plants.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

According to one aspect of the present invention there is provided a method of enhancing inorganic carbon fixation by a photosynthetic organism.

As used herein in the specification and in the claims section that follows the phrase "photosynthetic organism" includes organisms, both unicellular or multicellular, both prokaryotes or eukaryotes, both soil grown or aquatic, capable of producing complex organic materials, especially carbohydrates, from carbon dioxide using light as the source of energy and with the aid of chlorophyll and optionally associated pigments.

The method according to the present invention is effected by transforming cells of the photosynthetic organism with an expressible polynucleotide encoding a polypeptide having a bicarbonate (HCO_3^-) transporter activity.

As used herein in the specification and in the claims section that follows the term "transform" and its conjugations such as transformation, transforming and transformed, all relate to the process of introducing heterologous nucleic acid sequences into a cell or an organism. The term thus reads on, for example, "genetically modified", "transgenic" and "transfected" or "viral infected" and their conjugations, which may be used herein to further described the present invention. The term relates both to introduction of a heterologous nucleic acid sequence into the

genome of an organism and/or into the genome of a nucleic acid containing organelle thereof, such as into a genome of chloroplast or a mitochondrion.

As used herein in the specification and in the claims section that follows the phrase "expressible polynucleotide" refers to a nucleic acid sequence including a promoter sequence and a downstream polypeptide encoding sequence, the promoter sequence is so positioned and constructed so as to direct transcription of the downstream polypeptide encoding sequence.

As used herein in the specification and in the claims section that follows the term "polypeptide" refers also to a protein, in particular a transmembrane protein, which may include a transit peptide, and further to a post translationally modified protein, such as, but not limited to, a phosphorylated protein, glycosylated protein, ubiquitinylated protein, acetylated protein, methylated protein, etc.

As used herein in the specification and in the claims section that follows the phrase "bicarbonate transporter activity" refers to the direct activity of a membrane integrated protein in transporting bicarbonate across a membrane in which it is integrated. Such a membrane can be

the cell membrane and/or a membrane of an organelle, such as the chloroplast's outer and inner membrane. Such activity can be effected by direct expenditure of energy, i.e., ATP hydrolysis, which is available both in the cytoplasm and the chloroplast's stroma, or by co- or anti-transport, as effected by co- or antiporters while dissipating a concentration gradient of an ion across a membrane.

According to another aspect of the present invention there is provided a nucleic acid molecule for enhancing inorganic carbon fixation by a photosynthetic organism. The nucleic acid molecule according to this aspect of the present invention includes a polynucleotide encoding a polypeptide having a bicarbonate transporter activity.

As used herein in the specification and in the claims section that follows the term "nucleic acid molecule" includes polynucleotides, constructs and vectors. The terms "construct" and "vector" may be used herein interchangeably.

Such nucleic acid molecule or polynucleotide can be a nucleic acid sequence corresponding to at least a portion derived from SEQ ID NO:2, the portion encodes the protein having the bicarbonate transporter activity.

Alternatively or in addition it can be a nucleic acid sequence at least 60 %, preferably at least 65 %, more preferably at least 70 %, still more preferably at least 75 %, yet more preferably at least 80 %, more preferably at least 85 %, more preferably at least 90 %, yet more preferably at least 95 %, ideally 95-100 % identical to that portion, as determined using the Blast software where gap penalty equals 10 for existence and 10 for extension, average match equals 10 and average mismatch equals -5. It will be appreciated in this respect that SEQ ID NO:2 can be readily used to isolate homologous sequences which can be tested as described in the Examples section that follows for their bicarbonate transport activity. Methods for isolating such homologous sequences are extensively described in, for example, Sambrook *et al.* [9] and may include hybridization and PCR amplification.

Still alternatively or in addition it can be a nucleic acid segment hybridizable with that portion. Hybridization for long nucleic acids (e.g., above 200 bp in length) is effected according to preferred embodiments of the present invention by stringent or moderate hybridization, wherein stringent hybridization is effected by a hybridization solution containing 10 % dextrane sulfate, 1 M NaCl, 1 % SDS and 5 x 10^6 cpm ^{32}p labeled

probe, at 65 °C, with a final wash solution of 0.2 x SSC and 0.1 % SDS and final wash at 65°C; whereas moderate hybridization is effected by a hybridization solution containing 10 % dextrane sulfate, 1 M NaCl, 1 % SDS and 5 x 10^6 cpm ^{32}p labeled probe, at 65 °C, with a final wash solution of 1 x SSC and 0.1 % SDS and final wash at 50 °C.

Yet alternatively or in addition it can be a man induced variation, such as site directed or non-specific mutagenesis of that portion. Methods of man induced variation of nucleic acids are well known in the art and are further described in detail in reference [9].

Still alternatively or in addition it can be a naturally occurring variation of that portion. Such variations can be characterized and such sequence alterations isolated by one ordinarily skilled in the art using the assays and procedures described hereinunder in the Examples section that follows.

According to a preferred embodiment of the present invention the polypeptide encoded by the polynucleotide is at least 60 %, preferably at least 65 %, more preferably at least 70 %, still more preferably at least 75 %, yet more preferably at least 80 %, more preferably at least 85 %, more preferably at least 90 %, yet more preferably at least 95 %, ideally 95-100

% homologous (identical + similar) to SEQ ID NO:3 or a portion thereof having the bicarbonate transporter activity as determined using the Blast software where gap open penalty equals 11, gap extension penalty equals 1 and matrix is blosum62.

According to a preferred embodiment of the present invention, the polypeptide includes an N terminal transit peptide fused thereto which serves for directing the polypeptide to a specific membrane. Such a membrane can be, for example, the cell membrane, wherein the polypeptide will serve to transport bicarbonate from the apoplast into the cytoplasm, or, such a membrane can be the outer and preferably the inner chloroplast membrane, wherein the polypeptide will serve to transport bicarbonate from the cytoplasm to the intermembranal space and the stroma, respectively. Transit peptides which function as herein described are well known in the art. Further description of such transit peptides is found in, for example, Johnson *et al.* The Plant Cell (1990) 2:525-532; Sauer *et al.* EMBO J. (1990) 9:3045-3050; Mueckler *et al.* Science (1985) 229:941-945; Von Heijne, Eur. J. Biochem. (1983) 133:17-21; Yon Heijne, J. Mol. Biol. (1986) 189:239-242; Iturriaga *et al.* The Plant Cell (1989) 1:381-390; McKnight *et al.*, Nucl. Acid Res. (1990)

18:4939-4943; Matsuoka and Nakamura, Proc. Natl. Acad. Sci. USA (1991) 88:834-838. A recent text book entitled "Recombinant proteins from plants", Eds. C. Cunningham and A.J.R. Porter, 1998 Humana Press Totowa, N.J. describe methods for the production of recombinant proteins in plants and methods for targeting the proteins to different compartments in the plant cell. The book by Cunningham and Porter is incorporated herein by reference. It will however be appreciated by one of skills in the art that a large number of membrane integrated proteins fail to poses a removable transit peptide. It is accepted that in such cases a certain amino acid sequence in said proteins serves not only as a structural portion of the protein, but also as a transit peptide.

According to a preferred embodiment, the nucleic acid molecule further includes a plant promoter located upstream to the polynucleotide and being effective in expressing the polypeptide.

As used herein in the specification and in the claims section that follows the phrase "plant promoter" includes a promoter which can direct gene expression in plant cells (including DNA containing organelles). Such a promoter can be derived from a plant, bacterial, viral, fungal or animal origin. Such a promoter can be constitutive, i.e., capable of

directing high level of gene expression in a plurality of plant tissues, tissue specific, i.e., capable of directing gene expression in a particular plant tissue or tissues, inducible, i.e., capable of directing gene expression under a stimulus, or chimeric.

Thus, the plant promoter employed can be a constitutive promoter, a tissue specific promoter, an inducible promoter or a chimeric promoter.

Examples of constitutive plant promoters include, without being limited to, CaMV35S and CaMV19S promoters, FMV34S promoter, sugarcane bacilliform badnavirus promoter, CsVMV promoter, *Arabidopsis* ACT2/ACT8 actin promoter, *Arabidopsis* ubiquitin UBQ1 promoter, barley leaf thionin BTH6 promoter, and rice actin promoter.

Examples of tissue specific promoters include, without being limited to, bean phaseolin storage protein promoter, DLEC promoter, PHSβ promoter, zein storage protein promoter, conglutin gamma promoter from soybean, AT2S1 gene promoter, ACT11 actin promoter from *Arabidopsis*, napA promoter from *Brassica napus* and potato patatin gene promoter.

The inducible promoter is a promoter induced by a specific stimuli such as stress conditions comprising, for example, light, temperature,

chemicals, drought, high salinity, osmotic shock, oxidant conditions or in case of pathogenicity and include, without being limited to, the light-inducible promoter derived from the pea rbcS gene, the promoter from the alfalfa rbcS gene, the promoters DRE, MYC and MYB active in drought; the promoters INT, INPS, prxEa, Ha hsp17.7G4 and RD21 active in high salinity and osmotic stress, and the promoters hsr203J and str246C active in pathogenic stress.

There are various methods of introducing foreign genes into both monocotyledonous and dicotyledenous plants (Potrykus, I., Annu. Rev. Plant. Physiol., Plant. Mol. Biol. (1991) 42:205-225; Shimamoto *et al.*, Nature (1989) 338:274-276). The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include two main approaches:

(i) *Agrobacterium*-mediated gene transfer: Klee *et al.* (1987) Annu. Rev. Plant Physiol. 38:467-486; Klee and Rogers in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes, eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 2-25; Gatenby, in Plant

Biotechnology, eds. Kung, S. and Arntzen, C. J., Butterworth Publishers, Boston, Mass. (1989) p. 93-112.

(ii) direct DNA uptake: Paszkowski *et al.*, in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 52-68; including methods for direct uptake of DNA into protoplasts, Toriyama, K. *et al.* (1988) Bio/Technology 6:1072-1074. DNA uptake induced by brief electric shock of plant cells: Zhang *et al.* Plant Cell Rep. (1988) 7:379-384. Fromm *et al.* Nature (1986) 319:791-793. DNA injection into plant cells or tissues by particle bombardment, Klein *et al.* Bio/Technology (1988) 6:559-563; McCabe *et al.* Bio/Technology (1988) 6:923-926; Sanford, Physiol. Plant. (1990) 79:206-209; by the use of micropipette systems: Neuhaus *et al.*, Theor. Appl. Genet. (1987) 75:30-36; Neuhaus and Spangenberg, Physiol. Plant. (1990) 79:213-217; or by the direct incubation of DNA with germinating pollen, DeWet *et al.* in Experimental Manipulation of Ovule Tissue, eds. Chapman, G. P. and Mantell, S. H. and Daniels, W. Longman, London, (1985) p. 197-209; and Ohta, Proc. Natl. Acad. Sci. USA (1986) 83:715-719.

The *Agrobacterium* system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the *Agrobacterium* delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. Horsch *et al.* in Plant Molecular Biology Manual A5, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. A supplementary approach employs the *Agrobacterium* delivery system in combination with vacuum infiltration. The *Agrobacterium* system is especially viable in the creation of transgenic dicotyledenous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Following transformation plant propagation is exercised. The most common method of plant propagation is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in the crop, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. Basically, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the transformed plant be produced such that the regenerated plant has the identical traits and characteristics of the parent transgenic plant. Therefore, it is preferred that the transformed plant be regenerated by micropropagation which provides a rapid, consistent reproduction of the transformed plants.

Micropropagation is a process of growing new generation plants from a single piece of tissue that has been excised from a selected parent plant or cultivar. This process permits the mass reproduction of plants having the preferred tissue expressing the fusion protein. The new generation plants which are produced are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows mass production of quality plant material in a short period of time and offers a rapid multiplication of selected cultivars in the preservation

of the characteristics of the original transgenic or transformed plant. The advantages of cloning plants are the speed of plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. Thus, the micropropagation process involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During stage one, initial tissue culturing, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are produced to meet production goals. During stage three, the tissue samples grown in stage two are divided and grown into individual plantlets. At stage four, the transformed plantlets are transferred to a greenhouse for hardening where the plants' tolerance to light is gradually increased so that it can be grown in the natural environment.

The basic bacterial/plant vector construct will preferably provide a broad host range prokaryote replication origin; a prokaryote selectable

marker; and, for *Agrobacterium* transformations, T DNA sequences for *Agrobacterium*-mediated transfer to plant chromosomes. Where the heterologous sequence is not readily amenable to detection, the construct will preferably also have a selectable marker gene suitable for determining if a plant cell has been transformed. A general review of suitable markers for the members of the grass family is found in Wilmink and Dons, Plant Mol. Biol. Reptr. (1993) 11:165-185.

Sequences suitable for permitting integration of the heterologous sequence into the plant genome are also recommended. These might include transposon sequences and the like for homologous recombination as well as Ti sequences which permit random insertion of a heterologous expression cassette into a plant genome.

Suitable prokaryote selectable markers include resistance toward antibiotics such as ampicillin or tetracycline. Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art.

The constructs of the subject invention will include an expression cassette for expression of the fusion protein of interest. Usually, there will be only one expression cassette, although two or more are feasible.

The recombinant expression cassette will contain in addition to the heterologous sequence one or more of the following sequence elements, a promoter region, plant 5' untranslated sequences, initiation codon depending upon whether or not the structural gene comes equipped with one, and a transcription and translation termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

Viruses are a unique class of infectious agents whose distinctive features are their simple organization and their mechanism of replication. In fact, a complete viral particle, or virion, may be regarded mainly as a block of genetic material (either DNA or RNA) capable of autonomous replication, surrounded by a protein coat and sometimes by an additional membranous envelope such as in the case of alpha viruses. The coat protects the virus from the environment and serves as a vehicle for transmission from one host cell to another.

Viruses that have been shown to be useful for the transformation of plant hosts include CaV, TMV and BV. Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (BGV), EP-A 67,553 (TMV), Japanese Published Application No. 63-14693

(TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. *et al.*, Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988). Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants, is described in WO 87/06261.

Construction of plant RNA viruses for the introduction and expression of non-viral foreign genes in plants is demonstrated by the above references as well as by Dawson, W. O. *et al.*, Virology (1989) 172:285-292; Takamatsu *et al.* EMBO J. (1987) 6:307-311; French *et al.* Science (1986) 231:1294-1297; and Takamatsu *et al.* FEBS Letters (1990) 269:73-76.

When the virus is a DNA virus, the constructions can be made to the virus itself. Alternatively, the virus can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The virus can then be excised from the plasmid. If the virus is a DNA virus, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the virus is an RNA virus, the virus is

generally cloned as a cDNA and inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA virus is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

Construction of plant RNA viruses for the introduction and expression of non-viral foreign genes in plants is demonstrated by the above references as well as in U.S. Pat. No. 5,316,931

In one embodiment, a plant viral nucleic acid is provided in which the native coat protein coding sequence has been deleted from a viral nucleic acid, a non-native plant viral coat protein coding sequence and a non-native promoter, preferably the subgenomic promoter of the non-native coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral nucleic acid, and ensuring a systemic infection of the host by the recombinant plant viral nucleic acid, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native nucleic acid sequence within it, such that a fusion protein is produced. The recombinant plant viral nucleic acid may contain one or more additional non-native subgenomic

promoters. Each non-native subgenomic promoter is capable of transcribing or expressing adjacent genes or nucleic acid sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) nucleic acid sequences may be inserted adjacent the native plant viral subgenomic promoter or the native and a non-native plant viral subgenomic promoters if more than one nucleic acid sequence is included. The non-native nucleic acid sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral nucleic acid is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-native coat protein coding sequence.

In a third embodiment, a recombinant plant viral nucleic acid is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral nucleic acid. The inserted non-native subgenomic

promoters are capable of transcribing or expressing adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native nucleic acid sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that said sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral nucleic acid is provided as in the third embodiment except that the native coat protein coding sequence is replaced by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral nucleic acid to produce a recombinant plant virus. The recombinant plant viral nucleic acid or recombinant plant virus is used to infect appropriate host plants. The recombinant plant viral nucleic acid is capable of replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) in the host to produce the desired fusion protein.

Thus, according to a preferred embodiment of the present invention the polynucleotide or nucleic acid molecule of the present invention further includes one or more sequence elements, such as, but not limited to, a nucleic acid sequence encoding a transit peptide, an origin of replication for propagation in bacterial cells, at least one sequence element for integration into a plant's genome, a polyadenylation recognition sequence, a transcription termination signal, a sequence encoding a translation start site, a sequence encoding a translation stop site, plant RNA virus derived sequences, plant DNA virus derived sequences, tumor inducing (Ti) plasmid derived sequences and a transposable element derived sequence.

According to another preferred embodiment of the present invention, the step of transforming the cells of the photosynthetic organism with the expressible polynucleotide encoding the bicarbonate transporter is effected by a method such as genetic transformation and transient transformation. Genetic transformation can be effected by, for example, Agrobaterium mediated transformation, whereas transient transformation can be effected by, for example, viral transformation. Both transient and genetic transformation can be effected by

electroporation, particle bombardment or any of the other methods listed and further described hereinabove.

A technique for introducing heterologous nucleic acid sequences to the genome of the chloroplasts is known. This technique involves the following procedures. First, plant cells are chemically treated so as to reduce the number of chloroplasts per cell to about one. Then, the heterologous nucleic acid is introduced via particle bombardment into the cells with the aim of introducing at least one heterologous nucleic acid molecule into the chloroplasts. The heterologous nucleic acid is selected such that it is integratable into the chloroplast's genome via homologous recombination which is readily effected by enzymes inherent to the chloroplast. To this end, the heterologous nucleic acid includes, in addition to a gene of interest, at least one nucleic acid stretch which is derived from the chloroplast's genome. In addition, the heterologous nucleic acid includes a selectable marker, which serves by sequential selection procedures to ascertain that all or substantially all of the copies of the chloroplast genomes following such selection will include the heterologous nucleic acid. Further details relating to this technique are found in U.S. Pat. Nos. 4,945,050; and 5,693,507 which are incorporated

herein by reference. A polypeptide can thus be produced by the protein expression system of the chloroplast and become integrated into the chloroplast's inner membrane. It will be appreciated that cyanobacterial proteins are expected to express well by the chloroplast expression system.

According to another aspect of the present invention there is provided a transformed photosynthetic organism comprising the nucleic acid molecule or polynucleotide described herein.

According to a preferred embodiment of the present invention the photosynthetic organism is a plant, such as a crop plant. Preferably the plant is a C3 plant, such as, but not limited to, tobacco, tomato, soybeans, potato, cucumber, cotton, wheat, rice and barley because, as further detailed herein, such plants are more sensistive to limited water supply and/or low CO_2 atmosphere. However, over expressing C4 plants, such as, but not limited to, corn, sugar cane and sorghum are also expected to enjoy improved CO_2 fixation, especially under water-limiting conditions.

According to a preferred embodiment of the present invention the transformed photosynthetic organism is characterized by a photosynthetic rate at least 5 %, preferably at least 10 %, more preferably at least 15 %,

yet more preferably at least 25 %, still more preferably at least 30 %, more preferably at least 40 %, more preferably at least 50 %, ideally more preferably at least between 50 % and 100 % higher as compared to a control non-transformed organism under otherwise identical conditions. Such conditions and photosynthesis rate measurement procedures are further described in the Examples section that follows with respect to Table 2.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturers' specifications. These techniques and various other techniques are generally performed according to Sambrook et al. [9]. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Materials and Experimental Methods

Growth conditions:

Cultures of *Synechococcus* sp. strain PCC 7942 and mutant IL-2 thereof were grown at 30 °C in BG_{11} medium supplemented with 20 mM Hepes-NaOH pH 7.8 and 25 μg mL^{-1} kanamycin (in the case of the mutant). The medium was aerated with either 5 % v/v CO_2 in air (high CO_2) or 0.0175 % v/v CO_2 in air (low CO_2) which was prepared by

mixing air with CO_2-free air at a 1:1 ratio. *Escherichia coli* (strain DH5 α) were grown on an LB medium [9] supplemented with either kanamycin (50 μg/mL) or ampicillin (50 μg/mL) when required.

Measurements of photosynthesis and Ci uptake:

The rates of inorganic carbon (Ci)-dependent O_2 evolution were measured by an O_2 electrode as described elsewhere [10] and by a membrane inlet mass spectrometer (MIMS, [6, 11]). The MIMS was also used for assessments of CO_2 and HCO_3^- uptake during steady state photosynthesis [6]. Ci fluxes following supply of CO_2 or HCO_3^- were determined by the filtering centrifugation technique [10]. High-CO_2 grown cells in the log phase of growth were transferred to either low or high CO_2 12 hours before conducting the experiments. Following harvest, the cells were resuspended in 25 mM Hepes-NaOH pH 8.0 and aerated with air (Ci concentration was about 0.4 mM) under light flux of 100 μmol photon quanta m^{-2} s^{-1} Aliquots were withdrawn, immediately placed in microfuge tubes and kept under similar light and temperature conditions. Small amounts of ^{14}C-CO_2 or ^{14}C-HCO_3^- which did not affect the final Ci concentration, were injected, and the Ci uptake terminated after 5 seconds by centrifugation.

General DNA manipulations:

Genomic DNA was isolated as described elsewhere [12]. Standard recombinant DNA techniques were used for cloning and Southern analyses [12-13] using the Random Primed DNA Labeling Kit or the DIG system, (Boehringer, Mannheim). Sequence analysis was performed using the Dye Terminator cycle sequencing kit, ABI Prism (377 DNA sequencing Perkin Elmer). The genomic library used here was constructed using Lambda EMBL3/*Bam*HI vector kit (Stratagene, La Jolla, CA).

Construction and isolation of mutant IL-2:

A modification of the method developed by Dolganov and Grossman [14] was used to raise and isolate new high-CO_2-requiring mutants [4, 5]. Briefly, genomic DNA was digested with *Taq*I and ligated into the *Acc*I site of the polylinker of a modified Bluescript SK plasmid. The bluescript borne gene for conferring ampicillin resistance (within the *Sca*I site) was inactivated by the insertion of a cartridge encoding kanamycin resistance (Kan^r, [8]). *Synechococcus* sp. train PCC 7942 cells were transfected with the library [12]. Single crossover events which conferred Kan^r led to inactivation of various genes. The Kan^r cells

were exposed to low CO_2 conditions for 8 hours for adaptation, followed by an ampicillin treatment (400 μg/mL) for 12 hours. Cells capable of adapting to low CO_2 and thus able to grow under these conditions were eliminated by this treatment. The high-CO_2-requiring mutant, IL-2, unable to divide under low CO_2 conditions, survived, and was rescued following the removal of ampicillin and growth in the presence of high CO_2 concentration.

Cloning of the relevant impaired genomic region from mutant IL-2:

DNA isolated from the mutant was digested with *Apa*I located on one side of the *Acc*I site in the polylinker; with *Bam*HI or *Eco*RI, located on the other side of the *Acc*I site; or with *Mfe*I that does not cleave the vector or the Kan^r cartridge. These enzymes also cleaved the genomic DNA. The digested DNA was self-ligated followed by transfection of competent *E. coli* cells (strain DH5α). Kan^r colonies carrying the vector sequences bearing the origin of replication, the Kan^r cartridge and part of the inactivated gene were then isolated. This procedure was used to clone the flanking regions on both sides of the vector inserted into the mutant. A 1.3 Kbp *Apa*I and a 0.8 Kbp *Bam*HI fragments isolated from

the plasmids (one *Apa*I site and *Bam*HI site originated from the vector's polylinker) were used as probes to identify the relevant clones in an EMBL3 genomic library of a wild type genome, and for Southern analyses. The location of these fragments in the wild type genome (SEQ ID NO:1) is schematically shown in Figure 1. The *Apa*I fragment is between positions 1600 to 2899 (SEQ ID NO:1), marked as T and A in Figure 1; the *Bam*HI fragment is between positions 4125 to 4957 (SEQ ID NO:1) marked as B and T in Figure 1. The 0.8 Kbp *Bam*HI fragment hybridized with the 1.6 Kbp *Hinc*II fragment (marked E3 in Figure 1). The 1.3 Kbp *Apa*I fragment hybridized with an *Eco*RI fragment of about 6 Kbp. Interestingly, this fragment could not be cloned from the genomic library into *E. coli*. Therefore, the *Bam*HI site was used (position 2348, SEQ ID NO:1, Figure 1) to split the EMBL3 clone into two clonable fragments of 4.0 and 1.8 Kbp (E1 and E2, respectively, E1 starts from a *Sau*3A site upstream of the *Hind*III site positioned at the beginning of Figure 1). Confirmation that these three fragments were indeed located as shown in Figure 1 was obtained by PCR using wild type DNA as template, leading to the synthesis of fragments P1 and P2

(Figure 1). Sequence analyses enabled comparison of the relevant region in IL-2 with the corresponding sequence in the wild-type.

Experimental Results

Physiological analysis:

The IL-2 mutant grew nearly the same as the wild type cells in the presence of high CO_2 concentration but was unable to grow under low CO_2. Analysis of the photosynthetic rate as a function of external Ci concentration revealed that the apparent photosynthetic affinity of the IL-2 mutant was 20 mM Ci, which is about 100 times higher than the concentration of Ci at the low CO_2 conditions. The curves relating to the photosynthetic rate as a function of Ci concentration, in IL-2, were similar to those obtained with other high-CO_2-requiring mutants of *Synechococcus* PCC 7942 [16, 17]. These data suggested that the inability of IL-2 to grow under low CO_2 is due to the poor photosynthetic performance of this mutant.

High-CO_2-requiring mutants showing such characteristics were recognized among mutants bearing aberrant carboxysomes [9, 10, 12, 18, 19] or defective in energization of Ci uptake [20, 21]. All the carboxysome-defective mutants characterized to date were able to

accumulate Ci within the cells similarly to wild type cells. However, they were unable to utilize it efficiently in photosynthesis due to low activation state of rubisco in mutant cells exposed to low CO_2 [10]. This was not the case for mutant IL-2 which possessed normal carboxysomes but exhibited impaired HCO_3^- uptake (Table 1, Figures 4a-b). Measurements of ^{14}Ci accumulation indicated that HCO_3^- and CO_2 uptake were similar in the high-CO_2-grown wild type and the mutant (Table 1).

TABLE 1

	CO_2 Uptake		HCO_3^- Uptake	
	High CO_2	**Low CO_2**	**High CO_2**	**Low CO_2**
WT	31.6	53.9	30.9	182.0
IL-2	26.6	39.2	32.2	61.1

The rate of CO_2 and of HCO_3^- uptake in *Synechococcus* sp. PCC 79 and mutant IL-2 as affected by the concentration of CO_2 in the growth medium. The unidirectional CO_2 or HCO_3^- uptake of cells grown under high CO_2 conditions or exposed to low CO_2 for 12 hours is presented in μmole Ci accumulated within the cells mg^{-1} Chl h^{-1}. The results presented are the average of three different experiments, with four replicas in each experiment, the range of the data was within ±10 % of the average. WT - wild type.

Uptake of HCO_3^- by wild type cells increased by approximately 6-fold following exposure to low CO_2 conditions for 12 hours. On the other hand, the same treatment resulted in only up to a 2-fold increase in HCO_3^- uptake for the IL-2 mutant. Uptake of CO_2 increased by approximately 50 % for both the wild type and the IL-2 mutant following

transfer from high- to low CO_2 conditions. These data indicate that HCO_3^- transport and not CO_2 uptake was impaired in mutant IL-2.

The V_{max} of HCO_3^- uptake, estimated by MIMS [7, 22] at steady state photosynthesis (Figure 4a), were 220 and 290 μmol HCO_3^- mg^{-1} Chl h^{-1} for high- and low-CO_2-grown wild type, respectively, and the corresponding $K_{1/2}$ (HCO_3^-) were 0.3 and 0.04 mM HCO_3^-, respectively. These estimates are in close agreement with those reported earlier [7]. In high-CO_2-grown mutant IL-2, on the other hand, the HCO_3^- transporting system was apparently inactive. The curve relating the rate of HCO_3^- transport as a function of its concentration did not resemble the expected saturable kinetics (observed for the wild type), but was closer to a linear dependence as expected in a diffusion mediated process (Figure 4b). It was essential to raise the concentration of HCO_3^- in the medium to values as high as 25 mM in order to achieve rates of HCO_3^- uptake similar to the V_{max} depicted by the wild type.

The estimated V_{max} of CO_2 uptake by high-CO_2-grown wild type and IL-2 was similar for both at around 130-150 μmol CO_2 mg^{-1} Chl h^{-1} and the $K_{1/2}(CO_2)$ values were around 5 μM (Figures 4a-b), indicating that CO_2 uptake was far less affected by the mutation in IL-2. Mutant

cells that were exposed to low CO_2 for 12 hours showed saturable kinetics for HCO_3^- uptake suggesting the involvement of a carrier. However, the $K_{1/2}$ (HCO_3^-) was 4.5 mM HCO_3^- (i.e., 15- and 100-fold lower than in high- and in low-CO_2-grown wild type, respectively) and the V_{max} was approximately 200 μmol HCO_3^- mg^{-1} Chl h^{-1}. These data indicate the presence of a low affinity HCO_3^- transporter that is activated or utilized following inactivation of a high affinity HCO_3^- uptake in the mutant. The activity of the low affinity transporter resulted in the saturable transport kinetics observed in the low-CO_2-exposed mutant. These data further demonstrated that the mutant was able to respond to the low CO_2 signal.

The reason for the discrepancy between the data obtained by the two methods used, with respect to HCO_3^- uptake in wild type and mutant cells grown under high-CO_2-conditions, is not fully understood. It might be related to the fact that in the MIMS method HCO_3^- uptake is assessed as the difference between net photosynthesis and CO_2 uptake [6, 7, 22]. Therefore, at Ci concentrations below 3 mM, where the mutant did not exhibit net photosynthesis, HCO_3^- uptake was calculated as zero (Figures 4a-b). On the other hand, the filtering centrifugation technique, as used

herein, measured the unidirectional HCO_3^- transport close to steady state via isotope exchange, which can explain some of the variations in the results. Not withstanding, the data obtained by both methods clearly indicates severe inhibition of HCO_3^- uptake in mutant cells exposed to low CO_2. It is interesting to note that while the characteristics of HCO_3^- uptake changed during acclimation of the mutant to low CO_2, CO_2 transport was not affected (Figures 4a-b). It is thus concluded that the high-CO_2-requiring phenotype of IL-2 is generated by the mutation of a HCO_3^- transporter rather than in non-acclimation to low CO_2.

Genomic analysis:

Since IL-2 is impaired in HCO_3^- transport, it was used to identify and clone the relevant genomic region involved in the high affinity HCO_3^- uptake. Figure 1 presents a schematic map of the genomic region in *Synechococcus* sp. PCC 7942 where the insertion of the inactivating vector by a single cross over recombination event (indicated by a star) generated the IL-2 mutant. Sequence analysis (GenBank, accession No. U62616, SEQ ID NO:1) identified several open reading frames (identified in the legend of Figure 1), some are similar to those identified in *Synechocystis* PCC 6803 [23]. Comparison of the DNA sequence in

the wild type with those in the two repeated regions (due to the single cross over) in mutant IL-2, identified several alterations in the latter. This included a deletion of 4 nucleotides in the *Apa*I side and a deletion of 6 nucleotides but the addition of one bp in the *Bam*HI side (Figure 5). The reason(s) for these alterations is not known, but they occurred during the single cross recombination between the genomic DNA and the supercoiled plasmid bearing the insert in the inactivation library. The high-CO_2-requiring phenotype of mutant JR12 of *Synechococcus* sp. PCC 7942 also resulted from deletions of part of the vector and of a genomic region, during a single cross over event, leading to a deficiency in purine biosynthesis under low CO_2 [24].

The alterations depicted in Figure 5 resulted in frame shifts which led to inactivation of both copies of ORF467 (nucleotides 2670-4073 of SEQ ID NO:1, SEQ ID NO:2) in IL-2. Insertion of a Kan^r cartridge within the *Eco*RV or *Nhe*I sites in ORF467, positions 2919 and 3897 (SEQ ID NO:1), respectively (indicated by the triangles in Figure 1), resulted in mutants capable of growing in the presence of kanamycin under low CO_2 conditions, though significantly (about 50 %) slower than the wild type. Southern analyses of these mutants clearly indicated that

they were merodiploids, i.e., contained both the wild type and the mutated genomic regions.

Figures 2 and 3 show nucleic and amino acid alignments of ictB and slr1515, the most similar sequence to ictB identified in the gene bank, respectively. Note that the identical nucleotides shared between these nucleic acid sequences (Figure 2) equal 56 %, the identical amino acids shared between these amino acid sequences (Figure 3) equal 47 %, the similar amino acids shared between these amino acid sequences (Figure 3) equal 16 %, bringing the total homology therebetween to 63 % (Figure 3). When analyzed without the transmembrane domains, the identical amino acids shared between these amino acid sequences equal 40 %, the similar amino acids shared between these amino acid sequences equal 12 %, bringing the total homology therebetween to 52 %.

HCO_3^- transporters in Synechococcus sp. PCC 7942:

The protein encoded by ORF467 (SEQ ID NO:3) contains 10 putative transmembrane regions and is a membrane integrated protein. It is somewhat homologous to several oxidation-reduction proteins including the Na^+/pantothenate symporter of *E. coli* (Accession No.

P16256). Na^+ ions are essential for HCO_3^- uptake in cyanobacteria and the possible involvement of a Na^+/HCO_3^- symport has been discussed [3, 25, 26]. The sequence of the fourth transmembrane domain contains a region which is similar to the DCCD binding motif in subunit C of ATP synthase with the exception of the two outermost positions, replaced by conservative changes in ORF467. The large number of transport proteins that are homologous to the gene product of ORF467 also suggest that it is also a transport protein, possibly involved in HCO_3^- uptake. ORF467 is referred to herein as *ict*B (for inorganic carbon transport B [27]).

Sequence similarity between *cmpA,* encoding a 42-kDa polypeptide which accumulates in the cytoplasmic-membrane of low-CO_2-exposed *Synechococcus* PCC 7942 [28], and *nrtA* involved in nitrate transport [29], raised the possibility that CmpA may be the periplasmic part of an ABC-type transporter engaged in HCO_3^- transport [21, Omata, personal communication]. The role of the 42 kDa polypeptide, however, is not clear since inactivation of *cmpA* did not affect the ability of *Synechococcus* PCC7942 [30] and *Synechocystis* PCC6803 [21] to grow under a normal air level of CO_2 but growth was decreased under 20 ppm CO_2 in air [21]. It is possible that

Synechococcus sp. PCC 7942 contains three different HCO_3^- carriers: the one encoded by *cmpA*; IctB; and the one expressed in mutant IL-2 cells exposed to low CO_2 whose identity is yet to be elucidated. These transporters enable the cell to maintain inorganic carbon supply under various environmental conditions.

Transgenic plants transformed with ictB:

The coding region of *ictB* was cloned downstream of a strong promoter (CaMV 35S) and down stream to and in frame with the transit peptide of pea rubisco small subunit within a plasmid bearing a gene encoding kanamycin resistance, and suitable for the Agrobacterium mediated transformation of Arabidopsis. Transformation of Arabidopsis with this construct resulted in fast growing transgenic plants which were kanamycin resistant. Northern analysis performed on wild type and three transgenic plants (Figure 6) demonstrated that the transformants expressed the *ictB* whereas, as expected, the wild type did not.

Measurements of the photosynthetic rate with the aid of a gas exchange system indicated a considerably faster CO_2 fixation in the transgenic plants (Table 2). The improved rate of CO_2 fixation was mainly pronounced under low CO_2 concentrations such as experienced

within the air spaces in the leaves of crop plants under most environmental conditions. At higher CO_2 concentrations, where the rate of photosynthesis is CO_2-saturated and presumably independent of HCO_3^- transport, the wild type and transgenic plants exhibited similar photosynthetic rate, as expected. In a typical air space where the CO_2 concentration is 200-300 ppm, the photosynthetic rate of the transgenic plant was 24 to 42 % higher than in the wild type. At lower CO_2 concentrations such as effectively expected under water-limiting conditions the superiority of the transgenic plant was even more pronounced (Table 2).

TABLE 2

CO_2 Concentration (ppm)	Photosynthetic rate Wild Type (μmole CO_2 m^{-2} s^{-1})	Photosynthetic rate Transgenic plant (μmole CO_2 m^{-2} s^{-1})	Transgenic plant/ Wild Type
50	0.86	1.35	1.57
100	1.72	2.7	1.57
150	2.58	3.69	1.43
200	3.2	4.55	1.42
250	3.93	5.17	1.31
300	4.67	5.78	1.24
400	5.78	6.52	1.13
500	6.64	6.89	1.04
600	6.89	7.01	1.02

A comparison of the photosynthetic rate between the wild type and transgenic plants conducted with the aid of a CO_2 gas exchange analyzer (ADC, UK, Model LCA-2, Gas Exchange Unit).

It is thus expected that transgenic C3 plants, such as, but not limited to, tobacco, tomato, soybeans, potato, cucumber, cotton, wheat,

rice, barley and other C3 and also C4 crop plants, such as corn, sugar cane, sohrgum and others, expressing *ictB* will grow faster, and produce higher crop yield, including, for example, more and heavier fruits, especially under CO_2 and/or water limiting conditions. Tissue specific promoters, such as leaf specific promoters, could be used to highly express the *ictB* gene in leaves which are the primary photosynthetic organs in most plants.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

REFERENCES

1. Kaplan, A., Schwarz, R., Lieman-Hurwitz, J. and Reinhold, L. (1991) Plant Physiol. 97, 851-855.

2. Badger, M.R. and Price, D.G. (1994) Ann. Rev. Plant Physiol. Plant Mol. Biol. 45, 369-399.

3. Kaplan, A., Schwarz, R., Lieman-Hurwitz, J., Ronen-Tarazi, M. and Reinhold, L. (1994) in: The Molecular Biology of the Cyanobacteria (Bryant, D. Ed.), pp. 469-485, Kluwer Academic Pub., Dordrecht, The Netherlands.

4. Ronen-Tarazi, M., Schwarz, R., Bouevitch, A., Lieman-Hurwitz, J., Erez, J. and Kaplan, A. (1995) in: Molecular Ecology of Aquatic Microbes (Joint, I., Ed.), pp. 323-334. Springer-Verlag, Berlin.

5. Kaplan, A., Ronen-Tarazi, M., Zer, H., Schwarz, R., Tchernov, D., Bonfil, D.J., Schatz, D., Vardi, A., Hassidim, M. and Reinhold, L. (1998) Can. J. Bot. special issue, (In Press).

6. Sultemeyer, D., Price, G.D., Bryant, D.A. and Badger, M.R. (1997) Planta 201, 36-42.

7. Sultemeyer, D., Klughammer, B., Badger, M.R. and Price, G.D. (1998) Plant Physiol. 116, 183-192.

8. Ronen-Tarazi, M., Shinder, V. and Kaplan, A. (1998) FEMS Microbiol Lett. 159, 317-324.

9. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

10. Kaplan, A., Marcus, Y. and Reinhold, L. (1988) in: Methods in Enzymology (Packer, L. and Glazer, A.N., Eds.), pp. 534-539. Academic Press, New York.

11. Tchernov, D., Hassidim, M., Luz, B., Sukenik, A., Reinhold, L. and Kaplan, A. (1997) Curr. Biol. 7, 723-728.

12. Badger, M.R. and Price, G.D. (1992) Physiol. Plant. 84, 606-615.

13. Ronen-Tarazi, M., Lieman-Hurwitz, J., Gabay, C., Orus, M. and Kaplan, A. (1995) Plant Physiol. 108, 1461-1469.

14. Dolganov, N. and Grossman, A. (1993) J. Bacteriol. 175, 7644-7651.

15. Ronen-Tarazi, M., Bonfil, D.J., Schatz, D. and Kaplan, A. (1998) Can. J. Bot. special issue, (In Press).

16. Marcus, Y., Schwarz, R., Friedberg, D. and Kaplan, A. (1986) Plant Physiol. 82, 610-612.

17. Schwarz, R., Friedberg, D., Reinhold, L. and Kaplan, A. (1988) Plant Physiol. 88, 284-288.

18. Fukuzawa, H., Suzuki, E., Komukai, Y. and Miyachi, S. (1992) Proc. Natl. Acad. Sci. USA 89, 4437-4441.

19. Schwarz, R., Reinhold, L. and Kaplan, A. (1995) Plant Physiol. 108, 183-190.

20. Marco, M., Ohad, N., Schwarz, R., Lieman-Hurwitz, J., Gabay, C. and Kaplan, A. (1993) Plant Physiol. 101, 1047-1053.

21. Ohkawa, H., Sonoda, M., Katoh, H. and Ogawa, T. (1998) in: Proceedings of The Third International Symposium on Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms (Colman, B. Ed.), Can J Bot (in press).

22. Yu, J.W., Price, G.D. and Badger, M.R. (1994) Aust. J. Plant Physiol. 21, 185-195.

23. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T.,

Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. (1996) DNA Res. 3, 109-136.

24. Lieman-Hurwitz J., Schwarz, R., Martinez, F., Maor, Z., Reinhold, L., and Kaplan, A. (1990) in: Proceedings of The Second International Symposium on Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms (Colman, B. Ed.), Can J Bot 69: 945-950.

25. Kaplan, A., Schwarz, R., Ariel, R., and Reinhold, L. (1990) in: Regulation of Photosynthetic Processes (Kanay, R., Katoh, R.S. and Miyachi, S. Eds.), Special Issue of the Botanical Magazine, vol. 2, pp. 53-71, Tokyo.

26. Tyrrell, P. N., Kandasamy, R. A., Crotty, C. M. and Espie, G. S. (1996) Plant Physiol. 112, 79-88

27. Ogawa, T. (1991) Plant Physiol. 96, 280-284.

28. Omata, T. and Ogawa, T. (1986) Plant Physiol. 80, 525-530.

29. Omata, T. (1992) in: Research in Photosynthesis (Murata, N. Ed.) vol. III, 807-810, Kluwer Academic Pub., Dodrecht, The Netherlands.

30. Omata, T., Carlson, T.J., Ogawa, T. and Pierce, J. (1990) Plant Physiol. 93, 305-311.